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A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* Section *Flavi* isolated from Portuguese almonds

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ABSTRACT

A polyphasic approach consisting of morphological, chemical and molecular characterization was applied to 31 isolates of *Aspergillus* Section *Flavi* originating from Portuguese almonds, with the aim of characterizing and identifying aflatoxigenic and non-aflatoxigenic strains. On the basis of morphological characters (mainly colony color on Czapek-Dox agar and conidia morphology), we found two distinct groups among the population under study: 18 isolates (58%) had dark-green colonies and rough conidia, and were classified as *Aspergillus parasiticus*; the remaining 13 isolates (42%) had yellow-green colonies and smooth to finely rough globose conidia, and were classified as *Aspergillus flavus*. Chemical characterization involved the screening of the isolates for aflatoxins B (AFB) and G (AFG), and also for cyclopiazonic acid (CPA), by HPLC with fluorescence and UV detection, respectively. All *A. parasiticus* isolates were strong AFB and AFG producers, but no CPA production was detected, showing a consistent mycotoxigenic pattern. The *A. flavus* isolates showed to be more diversified, with 77% being atoxigenic, whereas 15% produced CPA and low levels of AFB and 8% produced the 3 groups of mycotoxins. Aflatoxin production was also screened on Coconut Agar Medium (CAM), and the results were consistent with the HPLC analysis. Sclerotia production showed no correlation to aflatoxigenicity.

Molecularly, two genes of the aflatoxin biosynthetic pathway, *aflD* (=*nor1*) and *aflQ* (=*ord1*=*ordA*) were tested for presence and expression (by PCR and RT-PCR, respectively). The presence of both genes did not correlate with aflatoxigenicity. *aflD* expression was not considered a good marker for differentiating aflatoxigenic from non-aflatoxigenic isolates, but *aflQ* showed a good correlation between expression and aflatoxin-production ability.

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1. Introduction

Aflatoxins, potent carcinogenic toxins, are the most widely studied of all mycotoxins. Aflatoxins frequently contaminate agricultural commodities, thus causing serious health hazards to humans and animals, as well as great economic loss. Although aflatoxin-production ability has been detected in various species of the *Aspergillus* genus, inside and outside the *Flavi* group, *Aspergillus flavus* and *Aspergillus parasiticus* remain the most important and representative aflatoxin producers occurring naturally in food commodities (Cary and Ehrlich, 2006). *Aspergillus nomius*, which has also been reported as a strong aflatoxin producer (Kurtzman et al., 1987), has rarely been identified in survey studies from agricultural soils and commodities (Ehrlich et al., 2007; Fiebelman et al., 1998; Ito et al., 1998; Razzaghi-Abyaneh et al., 2006). This can be an artefact resulting from its strong resemblance with *A. flavus* (Kurtzman et al., 1987), thus leading to misidentification.

The identification of *Aspergillus* Section *Flavi* has been traditionally based on morphological and biochemical characterization. Conidial wall ornamentation is regarded as the primary morphological diagnostic character for separation of *A. flavus* and *A. parasiticus*. Conidia of *A. flavus* have relatively thin walls which are finely to moderately roughened. Their shape can vary from spherical to elliptical. Conidia of *A. parasiticus* are more spherical and noticeably echinulate or spinulose. When grown on Czapek-Dox (CZ) colonies of *A. flavus* are yellow-green and those of *A. parasiticus* have a distinctly darker green (Klich, 2002; Samson et al., 2004). *A. nomius* is morphologically similar to *A. flavus* in colour, but conidia are more roughened (Kurtzman et al., 1987).

The mycotoxigenic profile (regarding aflatoxins B and G — AFB and AFG, respectively — and cyclopiazonic acid — CPA) of these strains has also been routinely used for identification purposes. In this matter, *A. parasiticus* tend to be more consistent than *A. flavus* (Wei and Jong, 1986), and therefore easier to classify. *A. parasiticus* strains are relatively uniform in their toxigenic abilities: they are usually strongly aflatoxigenic, producing both AFBs and AFGs, but not CPA. Non-aflatoxigenic strains have rarely been reported (Horn et al., 1996; Razzaghi-Abyaneh et al., 2006; Tran-Dinh et al., 1999; Vaamonde et al., 2003). On the other hand, *A. flavus* populations have been found to be extremely diverse in

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terms of morphology and toxigenicity, and have thus been divided into groups, depending on their toxigenic profile (Giorni et al., 2007; Razzaghi-Abyaneh et al., 2006; Vaamonde et al., 2003). Five groups have been proposed: (i) chemotype I for AFBs and CPA producers; (ii) chemotype II for AFBs, AFGs and CPA producers; (iii) chemotype III for AFBs producers; (iv) chemotype IV for CPA producers; and (v) chemotype V for non-producers (Vaamonde et al., 2003). The incidence of non-toxigenic strains of *A. flavus* has shown to be variable with geographic origin (Atehnkeng et al., 2008; Giorni et al., 2007; Razzaghi-Abyaneh et al., 2006) and substrate (Vaamonde et al., 2003). *A. nomius* exhibits a mycotoxigenic pattern similar to that of *A. parasiticus* (Kurztman et al., 1987).

Species identification based on morphological and biochemical characters is time-consuming and not always straight-forward, and molecular methods can be of help. But high genetic similarity between species of *Aspergillus* Section *Flavi*, as well as a high degree of intraspecific variability, has resulted in the inability to produce a molecular marker capable of consistently differentiating the various species. Some authors have reported the ability to differentiate *Aspergillus* Section *Flavi* species using molecular techniques (Ehrlich et al., 2004; Kumeda and Asao, 2001; Somashekar et al., 2004). These findings reflect the study of a limited number of strains (mostly lab strains), and they have not been tested with large numbers of field strains to confirm its utility as a molecular marker for species identification.

Molecular techniques have been widely applied also in the attempt of distinguishing aflatoxinogenic and non-aflatoxigenic strains of *A. flavus* and *A. parasiticus*, through the correlation of presence/absence of one or several genes involved in the aflatoxin biosynthetic pathway with the ability/inability to produce aflatoxins. Some groups have been able to distinguish these species from other food-borne fungi (Criseo et al., 2001; Färber et al., 1997; Geisen, 1996; Shapira et al., 1996), but none was capable of distinguishing aflatoxigenic from non-aflatoxigenic strains.

More recently, aflatoxin production and aflatoxigenic strains differentiation are being assessed by monitoring the expression of aflatoxin genes using the reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR methodologies. Such systems have been applied to monitor aflatoxin production and aflatoxin gene expression based on various regulatory and structural aflatoxin pathway genes in *A. parasiticus* and/or *A. flavus* (Degola et al., 2007; Mayer et al., 2003; Scherm et al., 2005; Sweeney et al., 2000), and were found to be very rapid and sensitive. But AF biosynthesis is based on a highly complex pathway. It requires at least 25 structural and 2 regulatory genes (Yu et al., 2004a), with possible alternative pathways (Detroy et al., 1973). It is thus not surprising that the protocols that can fully differentiate between AF producers and non-producers have not yet been successfully established. Furthermore, one has to be aware that some genes are not exclusive of the aflatoxin biosynthetic pathway, which could create false-positives from sterigmatocystin producing fungi (Paterson, 2006). An example being *Aspergillus nidulans*, which harbors the complete aflatoxin biosynthesis pathway except for the final step that converts sterigmatocystin to aflatoxin (Brown et al., 1996).

The identification methods previously described have not been applied in a concerted way. Also, they were mostly tested with lab strains. The aim of this study was to characterize 31 field strains of *Aspergillus* Section *Flavi* originating from Portuguese almonds, based on a polyphasic approach involving morphological, chemical and molecular patterns. This is the first report of characterization of *Aspergillus Flavi* isolated from a Portuguese agricultural commodity.

2. Materials and methods

2.1. Fungal isolates and culture conditions

Thirty-one isolates of *Aspergillus* Section *Flavi*, isolated from almonds from the Northeast of Portugal (region of Trás-os-Montes), were used in this study as field isolates, designated with the code yyAAspnn, where yy means the year, A refers to the commodity (almond), Asp refers to the genus *Aspergillus* and, nn is the isolate number. Type strains MUM92.01 (=NRRL6412, non-aflatoxigenic) and MUM92.02 (=NRRL3386, aflatoxigenic) from the collection of Micoteca of University of Minho (Portugal) were used as reference strains for *A. flavus* and *A. parasiticus*, respectively. Two *A. flavus* strains isolated from other commodities were included in the study: strain 01UAs55 (isolated from wine grapes, Portugal), and strain 05BrUAs01 (isolated from wine grapes, Brazil). These strains, although not being lab strains, were used as negative controls in molecular studies, since they were previously determined to be negative for the genes under study.

All isolates were maintained in 20% glycerol at −20 °C and grown on Malt Extract Agar (MEA: Malt 20 g/L, Glucose 20 g/L, Peptone 1 g/L, Agar 20 g/L) in the dark for 7 days at 25 °C whenever needed for further studies.

2.2. Morphological characterization

For each isolate, a loop full of spores was suspended in 500 µL of 0.2% agar, and this suspension was used for three-point inoculations on 9 cm diameter Petri dishes containing 20 mL of MEA and CZ (Sucrose 30 g/L, K_2HPO_4 1 g/L, $NaNO_3$ 2 g/L, KCl 0.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, $FeSO_4{\cdot}7H_2O$ 0.01 g/L, $ZnSO_4{\cdot}7H_2O$ 0.01 g/L, $CuSO_4{\cdot}5H_2O$ 0.005 g/L, Agar 20 g/L). Cultures were incubated for 7 days, in the dark, at 25 °C and then analysed for colony colour, presence and size of sclerotia, head seriation and conidia morphology. Colony colour on CZ was confirmed after 14 days of incubation. Identification followed the taxonomic keys and guides available for the *Aspergillus* genus (Klich, 2002; Samson et al., 2004). All isolates were cultured on *A. flavus* and *parasiticus* Agar (AFPA; Oxoid, Basingstoke, United Kingdom) for 3 to 5 days at 25 °C, in the dark, to confirm group identification by colony reverse colour. Isolates were also cultured on CZ at 42 °C, and colony diameter was measured after 7 days of incubation (Kurtzman et al., 1987), to confirm identification. Species identification followed characterization summarized in Table 1.

2.3. Mycotoxigenic ability of the isolates

2.3.1. Fluorescence on Coconut Agar Medium

For a preliminary screening of aflatoxin production, strains were inoculated at a central point on a 6 cm diameter Petri dish containing 10 mL of Coconut Agar Medium (Davies et al., 1987) and incubated for 7 days in the dark at 25 °C. Cultures were observed for fluorescence under long-wave UV light (365 nm) after 3, 5 and 7 days.

Table 1
A compilation of distinguishing characters of *Aspergillus* Section *Flavi* (from: Kurtzman et al., 1987; Klich, 2002; Samson et al., 2004)

Species	Seriation[a]	Conidia morphology	Colony color	AFPA	Colony diameter (cm), CZ, 42 °C	AFBs[b]	AFGs[b]	CPA[b]
A. flavus	b or b/u	Smooth	Yellow-green	Orange	2.4–3.6	+	−	+
A. parasiticus	u or u/b	Rough	Dark-green	Orange	1.8–3.3	+	+	−
A. nomius	u or u/b	Rough	Yellow-green to olive-green	Orange	0–1.5	+	+	−
A. oryzae	variable	Rough	Brown	Cream	1.8–3.2	−	−	(+)
A. sojae	−	Smooth	Pale brown	Orange	1.5–2.7	−	−	−
A. tamarii	−	Rough	Dark-brown	Brown	0.2–1.0	−	−	+

[a] u: uniseriate; b: biseriate; u/b: predominantly uniseriate; b/u: predominantly biseriate.
[b] +: presence; −: absence; (+) variable.

2.3.2. HPLC analysis

2.3.2.1. Aflatoxins detection. All strains were tested for aflatoxin production in aflatoxin-inducing Yeast Extract Sucrose (YES) medium (Yeast Extract 20 g/L, Sucrose 150 g/L, Agar 15 g/L). Strains MUM92.01, MUM92.02, 07AAsp05, 08AAsp42 and 08AAsp68 were also tested for aflatoxin production in the non-inducing Yeast Extract Peptone (YEP) medium (Yeast Extract 20 g/L, Peptone 150 g/L, Agar 15 g/L). Strains were inoculated on 6 cm diameter plates and incubated at 25–27 °C for 7 days, in the dark. Then the methodology of Bragulat et al. (2001) was employed: briefly, 3 agar plugs were removed from one colony, and placed into a 4 mL vial, where 1 mL of methanol was added. After 60 min, the extract was filtered by 0.45 µm filters and analysed by HPLC.

Samples were analysed using a HPLC equipped with a Jasco FP-920 fluorescence detector (365 nm excitation wavelength; 435 nm emission wavelength), using a photochemical post-column derivatization reactor (PHRED unit — Aura Industries, USA). Chromatographic separations were performed on a reverse phase C18 column (Waters Spherisorb ODS2, 4.6 mm×250 mm, 5 µm), fitted with a precolumn with the same stationary phase. The mobile phase used was pumped at 1.0 mL/min and consisted of an isocratic programme as follows: water:acetonitrile: methanol (3:1:1, v/v). The injection volume was 100 µL.

Aflatoxins standard was supplied by Biopure (Austria). A mix of aflatoxins, containing 2 µg/mL each of AFB1 and AFG1, and 0.5 µg/mL each of AFB2 and AFG2 was used. Samples were taken as positive for each of the toxins when yielding a peak at a retention time similar to each standard, with a height five times higher than the baseline noise.

2.3.2.2. Cyclopiazonic acid detection. The strains were tested for cyclopiazonic acid in Czapek Yeast Autolysate medium (CYA: Sucrose 30 g/L, Powdered Yeast Extract 5 g/L, K_2HPO_4 1 g/L, $NaNO_3$ 2 g/L, KCl 0.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, $FeSO_4{\cdot}7H_2O$ 0.01 g/L, $ZnSO_4{\cdot}7H_2O$ 0.01 g/L, $CuSO_4{\cdot}5H_2O$ 0.005 g/L, Agar 20 g/L). All strains were inoculated on 6 cm diameter plates and incubated at 25 °C for 14 days, in the dark (Gqaleni et al., 1997). Then the methodology of Bragulat et al. (2001) was employed, as already described for aflatoxin analysis.

Samples were analysed using a HPLC equipped with a Varian 2050 UV detector (285 nm). Chromatographic separations were performed on a EuroSpher 100 NH2 column (Knauer, 4.6 mm×250 mm, 5 µm), fitted with a precolumn with the same stationary phase. The mobile phase used was pumped at 1.0 mL/min and consisted of an isocratic programme as follows: acetonitrile:50 mM ammonium acetate (3:1, v/v), pH 5. The injection volume was 100 µL.

Cyclopiazonic acid standard was supplied by Sigma (St. Louis, MO, USA). Samples were taken as positive when yielding a peak at a retention time similar to the CPA standard, with a height five times higher than the baseline noise.

2.4. Molecular characterization

2.4.1. Detection of aflatoxin genes *aflD* (=*nor1*) and *aflQ* (=*ordA*=*ord1*)

The nomenclature of the genes will follow that proposed by Yu et al. (2004b).

2.4.1.1. DNA extraction. A loop full of spores was transferred from a 7 day old culture into a 15 mL tube containing 1.5 mL of lysis buffer (200 mM Tris–HCl pH 8.5; 250 mL NaCl; 25 mM EDTA; 0.5% [w/v] SDS) and approximately 1 g of sterile acid-washed 0.4- to 0.6-mm-diameter glass beads (Sigma, St. Louis, MO, USA), and vortexed for 5 min at maximum speed. Polysaccharides and proteins were precipitated by adding 750 µL of cold 3 M sodium acetate, pH 5.5. This mixture was gently mixed by inversion, placed at −20 °C for 10 min and centrifuged twice at 14000 g for 10 min (4 °C). Clean supernatant was then transferred to a new tube and precipitated with one volume of cold isopropanol (−20 °C). This solution was gently mixed by inversion for a few minutes, incubated at −20 °C for 1 h and centrifuged at 14000 g for 10 min (4 °C). DNA pellet was washed twice with 1.0 mL of cold 70% ethanol, centrifuged at 6000 g for 5 min (4 °C) and air dried. DNA was resuspended in 100 to 200 µL of ultra-pure water, depending on the yield, and stored at −20 °C.

2.4.2. PCR amplification

PCR amplifications were performed on 25 µL of a reaction mixture containing $MgCl_2$-free reaction buffer, 1.5 mM $MgCl_2$, 1.25 U of Taq polymerase, 200 µM of each dNTP, 0.2 µM of each primer (see Table 2 for list of primers) and 1 ng/µL of template DNA.

PCR was carried out as follows: 1) 1 step at 94 °C for 3 min; 2) 30 cycles of the following three steps: 1 min at 94 °C, 1 min at 55 °C, 1 min 72 °C; and 3) one final 10 min step at 72 °C.

Genes *aflD* and *aflQ* were tested for all strains, using the primer pairs nor1-F/nor1-R and ord1-gF/ord1-gR, respectively. *aflD* primers were specifically designed in this study; *aflQ* primers were selected from previous studies.

The housekeeping gene *tub1* coding for β-tubulin (primer pair tub1-F/tub1-R) was used as internal amplification control (IAC).

2.4.3. Analysis of *aflD* and *aflQ* expression

2.4.3.1. Total RNA extraction. For gene expression experiments, a loop full of spores was inoculated into a 50 mL tube containing 25 mL of YES broth and incubated for 4 days at 25 °C, in the dark with agitation. Strains MUM92.01, MUM92.02, 07AAsp05, 08AAsp42 and 08AAsp68 were also tested in the non-inducing medium YEP broth. Approximately 200 mg of mycelia were then recovered with a sterile spatula, dried in absorbent paper and ground with liquid nitrogen in a sterile, cold mortar and pestle. Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), including a step of genomic DNA digestion with RNase-free DNase set (Qiagen, Hilden, Germany), according to manufacturer's instructions. RNAs were aliquoted and stored at −80 °C.

2.4.3.2. Reverse transcriptase PCR. RT-PCR was performed in 20 µL reaction of 8 µL of One-Step RT-PCR Pre-Mix kit (INTRON Biotechnology, Gyeonggi-do, South-Korea), 0.2 µM of each primer (Table 2) and 1 µg of template RNA. Reverse Transcription was obtained at 45 °C for 30 min. PCR parameters followed those reported by Degola et al. (2007): 4 min at 94 °C; 60 s at 94 °C, 1 min at 60 °C and 1 min at 72 °C for 5 cycles; 1 min at

Table 2
Details of the target genes, primer sequences and expected product length in base pairs (bp) for PCR and RT-PCR

Primer pair	Gene	Primer sequence (5′→3′)	PCR product legth (bp)	RT-PCR product size (bp)	Reference
Tub1-F	tub1	GCT TTC TGG CAA ACC ATC TC	1406	1198	Scherm et al. (2005)
Tub1-R		GGT CGT TCA TGT TGC TCT CA			Scherm et al. (2005)
Nor1-F	aflD	ACC GCT ACG CCG GCA CTC TCG GCA C	400	400	This study
Nor1-R		GTT GGC CGC CAG CTT CGA CAC TCC G			This study
Ord1-gF	aflQ	TTA AGG CAG CGG AAT ACA AG			Sweeney et al. (2000)
Ord1-gR		GAC GCC CAA AGC CGA ACA CAA A	719	599	Sweeney et al. (2000)
Ord1-cR		GAATATCTGGACGTTTACCC	–	487	Degola et al. (2007)

94 °C, 1 min at 55 °C and 1 min at 72 °C for 30 cycles; and a final extension at 72 °C for 6 min. To check for the presence of genomic DNA contamination in the total RNA samples, PCR was carried out as described above, using the same set of primers and 1 µg of total RNA as template. The primers used for gene expression detection included those previously mentioned plus ord1-cR, specific for RNA.

The housekeeping gene *tub1* was used as IAC.

3. Results

3.1. Morphological analysis

Morphological characterization of the isolates is summarized in Table 3. On the basis of morphological characters (mainly colony color on CZ and conidia morphology), we found two distinct groups among the population under study: isolates with dark-green colonies and rough conidia, which were classified as *A. parasiticus* (18 isolates, 58%), and isolates with yellow-green colonies and smooth to finely rough globose conidia, classified as *A. flavus* (12 isolates, 42%). The isolate 07AAsp37 showed a somewhat distinct colony colour, more olivacious than those of the other *A. flavus* isolates, and moderately rough conidia.

Three of the 18 isolates classified as *A. parasiticus* (namely, 08AAsp34, 08AAsp36 and 08AAsp66) had limited growth on CZ at 42 °C, which could lead to the classification as *A. nomius*. But we were not able to find any other distinguishing features from other *A. parasiticus* isolates to support this reassignment. These isolates are all sclerotia producers. Kurtzman et al. (1987) characterize *A. nomius* sclerotia as vertically elongated, with indeterminate growth. This was not the case for these isolates, which showed globose to slightly elongate sclerotia typical of *A. flavus* and *A. parasiticus*.

All isolates were confirmed as *Aspergillus* Section *Flavi* by a bright orange colour of the colony reverse on AFPA (data not shown). None of the isolates showed a cream or brown colour on AFPA (corresponding to *A. oryzae* (Ahlburg) Cohn and *A. tamarii* Kita, respectively).

From the 31 isolates, 15 (48%) were able to produce dark oblong sclerotia bigger than 400 µm long in average, after 7 to 10 days of incubation on CZ (Table 3). Isolates of both *A. flavus* (8 isolates) and *A. parasiticus* (7 isolates) were sclerotia producers.

3.2. Chemical analysis

Analysis of aflatoxin production by fluorescence in CAM showed a good correlation with the HPLC results (Table 3). We found that all strains producing a strong signal for AFBs on the HPLC chromatogram showed a marked blue fluorescence pattern on CAM after 3 days of incubation, whereas those producing a weak signal by HPLC showed a

Table 3
Morphological and chemical characterization of isolates of *Aspergillus* Section *Flavi*

Isolate code	Morphology					Toxigenicity						Classification
	Sclerotia size (µm)[a]	Seriation[b]	Conidia[c]	Colony color[d]	Colony diameter at 42 °C[e]	Fluorescence on CAM	AF B1[f]	AF B2[f]	AF G1[f]	AF G2[f]	CPA[f]	
07AAsp05	860	b/u	r	d	1.4	Blue	+	+/−	+	+	−	*A. parasiticus*
07AAsp37	−	u/b	fr	y/d	1.4	Violet	+	+/−	+	+/−	+	*A. flavus*
07AAsp43	−	u/b	r	d	1.5	Blue	+	+/−	+	+	−	*A. parasiticus*
08AAsp34	600	u	r	d	0.8	Blue	++	+	+++	+	−	*A. parasiticus*
08AAsp35	500	b	s	y	2.7	−	−	−	−	−	−	*A. flavus*
08AAsp36	1100	u/b	r	d	0.5	Blue	++	+	+++	+	−	*A. parasiticus*
08AAsp37	1140	b	s	y	2.0	−	−	−	−	−	−	*A. flavus*
08AAsp38	−	u	r	d	1.3	Blue	++	+	+++	+	−	*A. parasiticus*
08AAsp39	−	u	r	d	2.2	Blue	++	+	++	+	−	*A. parasiticus*
08AAsp42	−	b	s	y	2.5	Violet	+	+/−	−	−	+	*A. flavus*
08AAsp43	−	b	s	y	2.1	−	−	−	−	−	−	*A. flavus*
08AAsp66	630	u/b	r	d	0.5	Blue	++	+	+++	+	−	*A. parasiticus*
08AAsp67	−	b/u	r	d	2.2	Green	+	+	++	++	−	*A. parasiticus*
08AAsp68	−	u	r	d	2.6	Blue	++	+	+++	+	−	*A. parasiticus*
08AAsp72	−	u	r	d	2.7	Blue	++	+	+++	+	−	*A. parasiticus*
08AAsp76	1220	b/u	s	y	1.5	−	−	−	−	−	−	*A. flavus*
08AAsp77	−	b	s	y	2.9	−	−	−	−	−	−	*A. flavus*
08AAsp83	480	u	r	d	1.6	Blue	++	+	+++	+	−	*A. parasiticus*
08AAsp101	−	u/b	r	d	1.6	Blue	++	+	+++	+	−	*A. parasiticus*
08AAsp103	−	u	r	d	1.7	Blue	++	+	+++	+	−	*A. parasiticus*
08AAsp105	970	b/u	s	y	3	−	−	−	−	−	−	*A. flavus*
08AAsp108	−	u	r	d	2.8	Blue	++	+	+++	+	−	*A. parasiticus*
08AAsp109	1070	b	s	y	2.7	−	−	−	−	−	−	*A. flavus*
08AAsp110	660	u/b	r	d	1.7	Blue	++	+	+++	+	−	*A. parasiticus*
08AAsp111	−	b/u	r	d	3.1	Blue	++	+	++	+	−	*A. parasiticus*
08AAsp112	1070	u/b	s	y	2.6	−	−	−	−	−	−	*A. flavus*
08AAsp113	1430	u/b	s	y	2.9	−	−	−	−	−	−	*A. flavus*
08AAsp115	980	b	s	y	2.0	−	−	−	−	−	−	*A. flavus*
08AAsp116	−	b	s	y	2.0	Violet	+	+/−	−	−	+	*A. flavus*
08AAsp117	920	u	r	d	1.8	Blue	++	+	+++	+	−	*A. parasiticus*
08AAsp158	−	u/b	r	d	2.3	Blue	+++	++	−/+	−/+	−	*A. parasiticus*
Controls												
MUM 92.01	440	b/u	s	y	1.8	Violet	+/−	−	−	−	+	*A. flavus*
MUM 92.02	560	u	r	d	n.d.	Blue	++	++	++	++	−	*A. parasiticus*
05BrUAs01	660	b	s	y	n.d.	−	−	−	−	−	−	*A. flavus*
01UAs55	n.d.	b	s	y	n.d.	−	−	−	−	−	−	*A. flavus*

[a] Size: average of 15 sclerotia; −: absence; n.d.: not determined.
[b] u: uniseriate; b: biseriate; u/b: predominantly uniseriate; b/u: predominantly biseriate.
[c] s: smooth; r: rough; fr: finelly rough.
[d] y: yellow-green; d: dark-green; y/d: in between y and d.
[e] Average of 3 colonies, in cm; n.d.: not determined.
[f] ++: strong signal; +: medium signal; +/−: weak signal; −/+: very weak signal; −: not detected.

Table 4
Incidence of chemotypes of *A. flavus* based on mycotoxigenic profile (aflatoxins and CPA)

Chemotype	Mycotoxins			Number of isolates of each chemotype (%)
	AFB	AFG	CPA	
I	+	−	+	2 (15%)
II	+	+	+	1 (8%)
III	+	−	−	0 (0%)
IV	−	−	+	0 (0%)
V	−	−	−	10 (77%)

(After Vaamonde et al., 2003).

weak violet fluorescence on CAM, only detectable after 5 days of incubation. Strain 08AAsp67, the only isolate for which both AFG1 and AFG2 signals were stronger than those for the AFBs on HPLC, showed a green fluorescence on CAM.

All *A. parasiticus* isolates showed a consistent mycotoxigenic profile: they all produced AFB and AFG, and no CPA production was detected. *A. flavus* isolates were assigned to 3 of the 5 chemotypes proposed by Vaamonde et al. (2003), as shown in Table 4: the vast majority (77%) were atoxigenic, whereas 2 isolates (15%) were CPA and AFB producers and one isolate (8%) produced the 3 groups of mycotoxins. Type-strain MUM 92.01, classified as non-aflatoxigenic, produced residual amounts of AFB1.

Table 5
Presence of genes *aflD* and *aflQ* (PCR) and their expression (RT-PCR) in *Aspergillus Flavi* isolates

Isolate code	Classification	AFB1	Gene presence (PCR)		Gene expression (RT-PCR)	
			aflD	*aflQ*	*aflD*	*aflQ*
07AAsp05	*A. parasiticus*	+	+	+	+	+
07AAsp37	*A. flavus*	+	+	+	+	+/−
07AAsp43	*A. parasiticus*	+	+	+	+	+
08AAsp34	*A. parasiticus*	++	+	+	+	+
08AAsp35	*A. flavus*	−	−	+	−	−
08AAsp36	*A. parasiticus*	++	+	+	n.d.	+
08AAsp37	*A. flavus*	−	+	+	+	−
08AAsp38	*A. parasiticus*	++	+	+	+	+
08AAsp39	*A. parasiticus*	++	+	+	+	+
08AAsp42	*A. flavus*	+	+	+	+	−
08AAsp43	*A. flavus*	−	+	+	+	−
08AAsp66	*A. parasiticus*	++	+	+	+	+
08AAsp67	*A. parasiticus*	+	+	+	n.d.	+/−
08AAsp68	*A. parasiticus*	++	+	+	n.d.	+
08AAsp72	*A. parasiticus*	++	+	+	n.d.	+
08AAsp76	*A. flavus*	−	+	+	n.d.	−
08AAsp77	*A. flavus*	−	+	+	n.d.	−
08AAsp83	*A. parasiticus*	++	+	+	n.d.	+
08AAsp101	*A. parasiticus*	++	+	+	n.d.	+
08AAsp103	*A. parasiticus*	++	+	+	n.d.	+
08AAsp105	*A. flavus*	−	+	+	n.d.	−
08AAsp108	*A. parasiticus*	++	+	+	n.d.	+
08AAsp109	*A. flavus*	−	+	+	n.d.	−
08AAsp110	*A. parasiticus*	++	+	+	n.d.	+
08AAsp111	*A. parasiticus*	++	+	+	n.d.	+
08AAsp112	*A. flavus*	−	+	+	n.d.	−
08AAsp113	*A. flavus*	−	+	+	n.d.	−
08AAsp115	*A. flavus*	−	+	+	n.d.	−
08AAsp116	*A. flavus*	+	+	+	n.d.	−
08AAsp117	*A. parasiticus*	++	+	+	n.d.	+
08AAsp158	*A. parasiticus*	+++	+	+	n.d.	+
Controls						
MUM 92.01	*A. flavus*	+/−	+	+	+	−
MUM 92.02	A. parasiticus	++	+	+	+	+
05BrUAs01	*A. flavus*	−	−	−	−	−
01UAs55	*A. flavus*	−	−	−	−	−

n.d. not determined.
+: strong signal; +/−: weak signal; −: no signal detected.

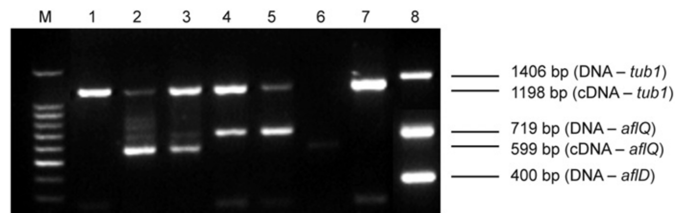

Fig. 1. Agarose gel electrophoretic pattern of reverse transcriptase-PCR products. M — molecular weight 100 bp ladder (Promega); 1 — 08AAsp35(−); 2 — 08AAsp36(+); 3 — 08AAsp72(+); 4 — 08AAsp76(−); 5 — 08AAsp77(−); 6 — 08AAsp83(+); 7 — 05BrUAs01 (−); 8 — DNA-PCR control.

3.3. Molecular analysis

PCR and RT-PCR results are presented in Table 5. From field isolates, and considering both genes under study (*aflD* and *aflQ*), only 08AAsp35 (−) was negative for the *aflD* amplicon, whereas 01UAs55 and 05BrUAs01, herein used as negative controls, showed no amplification for both *aflD* and *aflQ*. Gene expression analysis shows a more diverse pattern. All isolates tested for *aflD* expression gave a positive result, even for atoxigenic isolates. The only exceptions were, as expected, the isolates negative for *aflD* presence (08AAsp35, 01UAs55 and 05BrUAs01). *aflQ* expression was tested for all isolates. All strong AFB1 producers showed an amplicon near 600 bp, corresponding to the expected *aflQ* mRNA. This fragment was not detected in atoxigenic isolates. Among the weak producers of AFB1, isolates 07AAsp37 and 08AAsp67 showed a weak expression signal, and isolates 08AAsp42, 08AAsp116 and MUM92.01 showed no signal for *aflQ* expression. Fig. 1 is representative of the electrophoretic band patterns obtained for both aflatoxigenic and non-aflatoxigenic isolates relative to *tub1* and *aflQ* expression.

4. Discussion

In the present study, we aimed to identify and characterize 31 *Aspergillus* isolates belonging to Section *Flavi*. Morphological differentiation of species belonging to this group is difficult, due not only to interspecific similarities, but also to intraspecific variability. The major morphological characters used for *A. flavus* and *A. parasiticus* distinction are colony color and conidia morphology, but sclerotia presence and size as well as conidial head seriation are other characters that can be used to assist identification. Morphological characterization is usually complemented with mycotoxigenic pattern of AFs and CPA production ability. Kurtzman et al. (1987) refer to an extra character capable of distinguishing A. *nomius* from other related species, which is its limited growth at 42 °C on CZ.

Kozakiewicz (1989) reported that production of sclerotia is a rare characteristic of *A. flavus* strains only, and, in accordance to Klich (2007), the presence of sclerotia *per se* does not seem to be related to aflatoxin production, but the presence of small sclerotia appears to be correlated with high aflatoxin production. Several authors have tried to establish a correlation between sclerotia production ability and aflatoxigenicity, but published data are contradictory. Various studies refer to a positive correlation between high aflatoxin production and presence of small sclerotia (Chang et al., 2001; Cotty, 1989, 1997; Novas and Cabral, 2002; Pildain et al., 2004), whereas others report no correlation between sclerotial production/size and aflatoxigenicity (Giorni et al., 2007; Razzaghi-Abyaneh et al., 2006) or even an inverse correlation, with L-type strains being the most toxigenic (Abbas et al., 2005). Inconsistency of results may impart from the fact that fungal growth conditions have not been standardized and several culture media have been used for this purpose. In accordance to Cotty (1989), S-type strains usually produce high levels of aflatoxins and numerous sclerotia smaller than 400 µm in diameter. One atypical S-type *A. flavus* producer of AFBs, AFGs and CPA have been raised to species and named *A. parvisclerotigenus* (Frisvad et al., 2005).

In our study, we have identified both *A. parasiticus* and *A. flavus* isolates, aflatoxigenic and non-aflatoxigenic, as sclerotia producers. Among the *A. flavus* isolates, we could only detect the L-morphology, since they all produced sclerotia bigger than 400 µm under the tested conditions. We could not establish a correlation between sclerotia presence/size and toxigenicity. Isolate 07AAsp37, which produces both AFs and CPA did not produce small sclerotia, a condition required to be classified as *A. parvisclerotigenus* (Frisvad et al., 2005), and it remained with the classification *A. flavus*.

It has been reported that CAM fluorescence does not always correspond to aflatoxin detection by chromatography (Abarca et al., 1988; Giorni et al., 2007; Scherm et al., 2005). Abarca et al. (1988) report that blue fluorescence on CAM was detected in only 4 out of ten aflatoxigenic *A. flavus* strains. We found that all isolates producing a strong signal for AFBs on the HPLC chromatogram showed a marked blue fluorescence pattern (all of them *A. parasiticus*), whereas those producing a weak signal on HPLC showed weak violet fluorescence on CAM (all being *A. flavus*). The only isolate for which the AFGs signal was stronger than that for the AFBs on HPLC, showed a green fluorescence on CAM.

The usually accepted formula is that not all *A. flavus* isolates produce aflatoxins, and those that do usually produce only AFBs (and CPA), whereas almost all *A. parasiticus* isolates produce both aflatoxins B and G, but not CPA (Klich, 2007). But numerous studies have shown that the mycotoxigenic potential and profile of *A. flavus* is far more variable. In fact, this species has been frequently divided into groups, depending on their toxigenic profile (Giorni et al., 2007; Razzaghi-Abyaneh et al., 2006; Vaamonde et al., 2003). The incidence of atoxigenic strains of *A. flavus* has shown to be highly variable with geographic origin (Atehnkeng et al., 2008; Pildain et al., 2004; Razzaghi-Abyaneh et al., 2006) and substrate (Vaamonde et al., 2003).

In the present study, we found a vast majority (77%) of atoxigenic *A. flavus* isolates. Aflatoxigenic ability is, in fact, an unstable character in *A. flavus*, and its adaptation to the carbon-rich environments of certain agricultural commodities may be involved in gene loss responsible for the loss of aflatoxigenicity (Perrone et al., 2007). This could possibly be the case for our substrate.

A. parasiticus strains are, as already mentioned, more uniform in their toxigenic abilities: they are usually reported as strongly aflatoxigenic, and are rarely non-aflatoxigenic (Horn et al., 1996; Razzaghi-Abyaneh et al., 2006; Tran-Dinh et al., 1999; Vaamonde et al., 2003). With the exception of one isolate (08AAsp67), all our *A. parasiticus* isolates were found to be strongly aflatoxigenic.

Our results are similar to those obtained in a survey in corn field soils in Iran. Razzaghi-Abyaneh et al. (2006) report 100% aflatoxigenic *A. parasiticus* isolates and only 27.5% of aflatoxigenic *A. flavus* strains.

Although type-strain MUM92.01 (NRRL 6412) is classified as non-aflatoxigenic, we detected a weak AFB1 production ability. In fact, Wicklow et al. (1981) report that single-spore isolates of this strain were either AFB1 producers or non-producers on aflatoxin-production ability (APA) medium, and that it produced substantial amounts of aflatoxin when cultivated on cracked corn. These results provide evidence that strains can be genetically heterogeneous and that *in vitro* conditions can be misleading.

For the molecular analysis of our isolates, we have selected the *aflD* gene, which is responsible for the conversion of norsolorinic acid (NOR) to averantin (AVN) in the middle of the aflatoxin biosynthetic pathway (Yu et al., 2004a), because its expression had been reported as showing a high correlation to aflatoxigenic ability (Scherm et al., 2005). The *aflQ* gene was specifically chosen because it is considered to be the only gene involved in the final step of transforming *O*-methylsterigmatocystin (OMST) into AFB1, a crucial step of the aflatoxin pathway that seems to be unique to aflatoxigenic species (Prieto and Woloshuk, 1997).

As expected, the presence of these two genes could not be correlated to aflatoxin producing ability. Other authors had already tried multiplex PCR, without success (Geisen, 1996).

Scherm et al. (2005) tested 9 structural and 2 regulation genes in 13 lab strains and concluded that *aflD* expression had the best correlation between aflatoxigenicity and gene expression, and that *aflQ* expression did not show any consistency. Furthermore, they could not identify *aflQ* expression in any of the *A. flavus* strains, only in the aflatoxigenic *A. parasiticus* strains.

Since multiplex RT-PCR for the 3 genes (*tub1*, *aflQ* and *aflD*) revealed some inconsistency in the amplification patterns, we chose to test *aflD* and *aflQ* expression separately. We found expression of *aflD* in both aflatoxigenic and non-aflatoxigenic isolates, and for that reason we chose not to analyse its expression for all the isolates.

In respect to *aflD* expression, our results are contradictory to those reported by these authors. We could not find any correlation between *aflD* expression and aflatoxin production.

RT-PCR for *aflQ* showed a confusing, but consistent, band pattern (Fig. 1). When using the primer pair ord1-gF/ord1-gR proposed by Scherm et al. (2005) for the amplification from RNA, we detected a band corresponding to the size of the expected amplification from DNA (719 bp) in all isolates and another band corresponding to the expected amplification from RNA (599 bp) in the toxigenic isolates. To exclude the possibility of DNA contamination of the RNA, we ran a PCR for the RNA samples and no amplification occurred, confirming the efficacy of the DNase treatment. Furthermore, if any DNA contamination was to be present, two bands for the *tub1* gene would appear at sizes 1406 bp (DNA) and 1198 bp (RNA). Only the smaller band was present, further confirming the purity of the RNA samples. The primer pair ord1-gF/ord1-cR proposed by Degola et al. (2007) did not produce any amplicon.

We detected a fragment corresponding to *aflQ* mRNA (599 bp) in all strong aflatoxigenic isolates, but not in the non-aflatoxigenic nor in the weak AFs producers (all *A. flavus*). Scherm et al. (2005) were able to detect *aflQ* expression in *A. parasiticus* strains only, and not in any of the *A. flavus* tested, even the aflatoxigenic ones. Our strong aflatoxigenic isolates, which showed a marked *aflQ* signal, are all classified as *A. parasiticus*. The fact that we were not able to detect *aflQ* expression in aflatoxigenic *A. flavus* is in accordance with those authors. It could result from the fact that they are very weak AFs producers, and gene expression is not detected, or because of incompatibility of the primers with *A. flavus aflQ* mRNA sequence. In fact, Sweeney et al. (2000) tested this primer pair for one strain of *A. parasiticus* only, but the EMBL sequence used for constructing the primers is reported to be the one corresponding to the *A. flavus aflQ* DNA sequence (=*ord1*, EMBL Accession no. U81806). Primers sequences are in fact present in both *A. flavus aflQ* DNA and cDNA (EMBL Accession no. U81807), so the non-detection of *A. flavus aflQ* expression should not be the result of lack of complementarity between mRNA and primers sequences. However, we could detect a weak signal in the weak producer 07AAsp37, classified as *A. flavus*.

In conclusion, a polyphasic approach consisting of morphological, chemical and molecular characterization was applied to 31 isolates of *Aspergillus* Section *Flavi* originating from Portuguese almonds with the aim of characterizing and identifying aflatoxigenic and non-aflatoxigenic strains. Fifty-eight percent were *A. parasiticus*, all being aflatoxin producers, and 42% *A. flavus*, of which only 23% were aflatoxigenic. Mycotoxin production on CAM revealed consistent to HPLC analysis, and we could even differentiate a strong AFGs producer by a green fluorescence on CAM. Molecularly, *aflD* expression was not considered a good marker for differentiating aflatoxigenic from non-aflatoxigenic isolates, but *aflQ* showed a good correlation between expression and aflatoxin-production ability.

All material having had contact with mycotoxins was considered hazardous waste, and was thus conveniently decontaminated prior to disposal.

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